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SMALL BUSINESS INNOVATION RESEARCH PROGRAM
PHASE 1 – FY 1987
PROJECT SUMMARY

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Proposal Title
Chemotherapy of Leishmania with Oligodeoxynucleotide Probes

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Technical Abstract (Limit your abstract to 200 words with no classified or proprietary information/data.)

The goal of this project has been to investigate the potential of antisense oligonucleotides to kill *Leishmania enriettii* cells in culture. These antisense oligonucleotides are directed against the 35-base leader sequence spliced to the 5'-end of all *Leishmania* mRNA, with the objective of achieving cell kill by arresting translation. Agents that successfully arrested growth or killed the parasites *in vitro* would be viable candidates for study in animal models for treatment of Leishmaniasis, a major problem for American military personnel in certain tropical regions. We have found that mRNA translation can be significantly inhibited by antisense oligonucleotides 20 nucleotides long, and that this is improved by addition of lipophilic or planar aromatic moieties on the 5'-end of the oligonucleotide. These modified oligonucleotides have shown sequence-specific cell killing ability at concentrations at a concentration of 100 μM. In this project we have sequenced, for the first time, the spliced leader sequences of *L. mexicana amazonensis* and *L. braziliensis guyanensis*, two species pathogenic to humans, and shown them to be identical to that of *L. enriettii*. We have also demonstrated that we can arrest translation of mRNA from these species.

Anticipated Benefits/Potential Commercial Applications of the Research or Development

A successful program to develop antisense oligonucleotides capable of killing *Leishmania* parasites in culture would likely lead to a selective, nontoxic drug for the treatment of all of the *Leishmania* species pathogenic to humans.

List a maximum of 8 Key Words that describe the Project.

Oligonucleotide, antisense, Leishmania, acridine, translation, mRNA

I. Introduction

The research covered by this contract was to demonstrate the potential for the use of oligodeoxynucleotides (hereinafter called oligonucleotides or probes) as antileishmanial agents. The sequence of these probes is antisense to the spliced leader sequence of certain mRNA in *Leishmania enriettii*. The following specific objectives were proposed:

Objective 1

To demonstrate arrest of translation of *L. enriettii* tubulin mRNA's by oligodeoxynucleotides complementary to the leader sequence.

Objective 2

To demonstrate that cell growth of *L. enriettii in vitro* could be arrested by these oligodeoxynucleotides, and to determine the effect of oligodeoxynucleotide length and concentration on that cytotoxicity.

Objective 3

To demonstrate that the addition of a covalent crosslinking group to the oligodeoxynucleotide results in improvement of its efficacy as an inhibitor of cell-free translation, and as a cytotoxic agent in cell culture.

Objective 3 was not addressed in the course of this work due to the length of time required to establish the cellculture system and verify the results from the rest of the oligonucleotides and controls.

II. Methods

A. Synthesis of Oligonucleotides

The list of oligonucleotides synthesized for this project is shown in Appendix I. Most of these oligonucleotides are antisense (or sense controls) to a portion of the 35-base leader sequence of *L. enriettii* mRNA; some are antisense to splice junctions on the medRNA or to the splicing apparatus itself. These oligonucleotides were selected to examine the effects of oligonucleotide length and target sequence, and of the effect of modifications to the 5'-terminus. The latter modifications could aid nuclease resistance and cell uptake as well as hybrid stability.

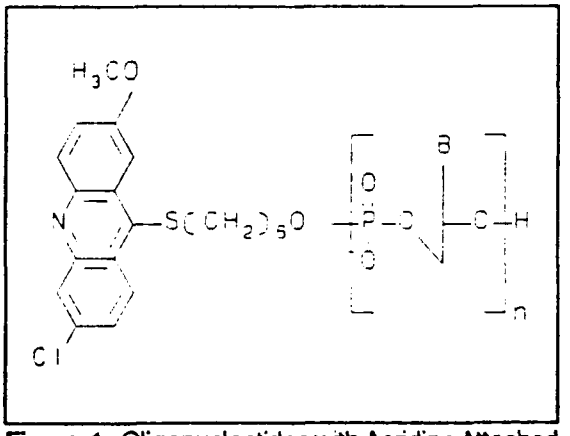

Figure 1. Oligonucleotides with Acridine Attached to the 5'-End

New lots of several nucleotides have been synthesized in the course of the project; a few unreliable batches, notably of LE15 and LE31, were found initially. We have found that the T_m values obtained as described below are good indicators of integrity of oligonucleotide

preparations.

We and others have found, as explained in our original application, that attachment of an intercalating agent (in this case, acridine) to either terminus of an oligonucleotide gives an increase in hybrid stability, in stability to enzymatic degradation, and possibly in cell uptake. We have designed and prepared a proprietary reagent for attaching a tethered acridine to the 5'-terminus of an oligonucleotide on the automated synthesizer, and the generic structure of oligonucleotides bearing this modification is shown in Figure 1.

There is evidence that antisense oligodeoxynucleotides having a methanephosphonic acid residue in place of the phosphoric acid in the normal DNA backbone, and which are therefore nonionic, are better inhibitors of mRNA translation in cells in culture, and we wanted to examine the effect of this structural modification in our system. These are denoted "MP" in the name code in Appendix 1, and the structure of the internucleotide linkage is shown in Figure 2.

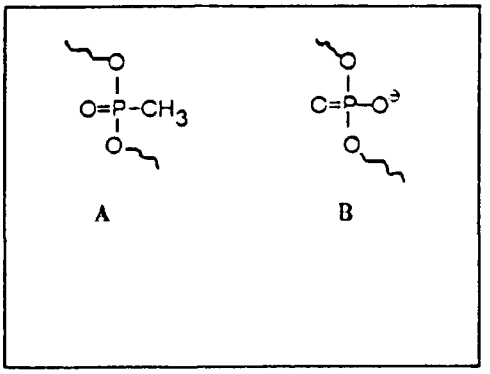

Figure 2. Comparison of the Methyl Phosphonate Linkage (A) with the Normal Phosphodiester Linkage (B) in Oligonucleotides.

We have developed a new reagent which can be used to attach a cholesterol group at the 5'-terminus of an oligonucleotide. There have been reports at recent conferences that indicate that this modification aids cell penetration. Its structure is shown in Figure 3.

B. Determination of T_m of New Oligonucleotides

We use a microprocessor-controlled Gilford 2600 spectrophotometer with a thermo-programming unit to determine the T_m's of our oligonucleotides by monitoring the hyperchromic UV change from duplexes to single strands in the presence of reverse complimentary sequences. Purity of oligonucleotides can be determined by this method, as well as the effect of chemical modifications (favorable or unfavorable) on the stability of oligonucleotide-DNA duplexes.

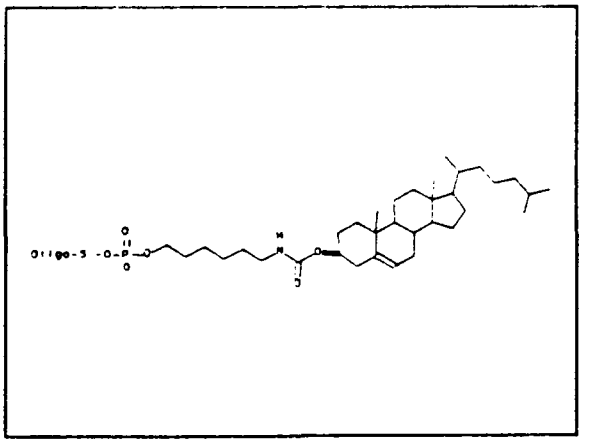

Figure 3. Oligonucleotide with cholesterol tethered to the 5'-position.

C. Establishment and Maintenance of Leishmania Cultures

We have obtained a culture of *L. enriettii* from Dr. S. Landfear of the Oregon Health Sciences Center. Viable cultures of *L. enriettii* were established in our facility, and the effect of serum concentration on clustering and division time of the promastigotes was investigated. The division time of our *L. enriettii* cell population was dependent on the amount of fetal calf serum (FCS) present. In a 20% FCS medium, the division time was about 8 hrs. In a 5-10% FCS medium, the division time varied from 12 to 24 hrs. The

cells appeared to be in log growth up to 1-2 x 10^7 cells/ml. The cells were irreversibly stressed at levels higher than 2 x 10^7 cells/ml. In defined media the cells survived up to a week. Clustering of cells seems to be best controlled by shaking the growing cultures.

For cytoxicity assays, cells were grown at 26° C. Aliquots of 100 μL of cells at a concentration of 1x10^6 cells/mL were added to microtiter plate wells and varing amounts of stock oligonucleotide (stock concentration = 2 mM) were added. Water was added to a total volume of 125 μL, and the cells were incubated for 48 hr. 10 μL aliquots of these cultures were counted by visual examination under the microscope.

D. Inhibition of Cell-free Translation

Cells were grown on a large scale and harvested, and a large quantity (4 mg) of RNA was isolated for translation inhibition experiments.

E. RNA Sequencing

To verify the sequence of the spliced leader in related species of Leishmania, a DNA dodecamer complimentary to the consensus 3'-terminal sequence of the spliced leader was prepared and annealed to the poly(A) mRNA fraction derived from individual Leishmanial cultures. The primed RNA was sequenced by the Sanger protocol using dideoxynucleotide triphosphates in the presence of reverse transcriptase, as previously described.[1]

III. Results

A. Effect of Oligonucleotide Modifications on Hybrid Stability

Spectrophotometric analysis was conducted in a solution containing PBS with a concentration of oligo and target each at 2 uM. Thus, T_m values are not absolute but are to be used for relative comparisons.

We have found that the T_m value of a current batch of LE15 was 2° C below the unmodified oligonucleotide of the same sequence (LE1). We know from previous data using a filter method that the T_m of these acridine modified oligonucleotides should be 5° C above the unmodified sequence, and that the results obtained spectrophotometrically agree with the filter method. We apparently have one bad batch or decomposition of LE15. The T_m of LE 24, a 20 mer with an acridine tail which has shown activity, is 3° degrees higher than the unmodified sequence. The T_m of a newly synthesized LE 15 is elevated by 3.5°, as expected.

The following conclusions may be drawn about the hybridization properties of the modified oligonucleotides:

- Acridine raises the relative Tm of an oligonucleotide duplex by approximately 3°, 6°,

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[1]. Scholler, J. K., Aline, Jr., R. F., and Stuart, K. D. (1988). *Mol. Biochem. Parisitol.*, **29**, 89-104.

Table 1. Effect of length and substituent at the 5'-position on the T_m value of oligonucleotides, when hybridized with complementary strands of various lengths.

Code Name	Modification at 5'-terminus	Oligo Length	Target Length	T_m
LE1	None	12	35	41.6
LE15	Acridine	12	35	44.8
LE32	Cholesterol	12	35	43.8
LE1	none	12	12	39.0
LE32	Cholesterol	12	12	39.0
LE5	None	16	35	47.6
LE34	Acridine	16	35	53.4
LE5	None	16	16	48.8
LE34	Acridine	16	16	54.8
LE7	None	20	35	57.0
LE24	Acridine	20	35	60.8

All Values ± 0.6°C

and 4° when attached to the 5' terminus of a 12, 16, and 20 mer, respectively. This increase in stability appears to be independent of the target length, and is consistent with intercalating into the duplex.

- Cholesterol tailed oligonucleotides appeared to raise the relative T_m of the 12 mer duplex by 2° when the 35 nucleotide target was used, but not when the target was the same length. This suggests that the cholesterol moiety interacts with the single-stranded overhang of the target.

- T_m evaluation of every modified oligonucleotide is a necessary quality control test. It detects inferior batches of oligos which can not be detected by simple gel or chromatographic analysis.

B. Inhibition of mRNA Translation

The reticulocyte cell-free translation assay for Leishmanial RNA has been optimized with necessary K and Mg ions. Translational inhibition experiments have been completed (Tables 2 and 3). Table 2 shows that the normal phosphodiesters inhibit at lengths of 16 nucleotides (nt) and greater (LE 5,7,9). The 35 nt oligonucleotide (LE 9) gives the greatest inhibition. The 12 nt methylphosphonates are strong inhibitors of translation being more effective than longer phosphodiester oligonucleotides. The acridine tailed oligonucleotides gave mixed results, with the 5' most 12 nt oligonucleotide showing no effect (LE15) nor the 3' most 12 nt oligonucleotide (LE 16) but more internal sequences suggesting inhibition (LE 28 and 30). The cholesterol tailed oligonucleotide gave strong inhibition at 50 μM, but the lower concentration needs repeating. Studies are continuing with appropriate controls to ascertain any *non*-antisense effects each oligonucleotide may have on translation,

Table 2

Effect of oligonucleotides on cell-free translation of L. Enriettii RNA.

Oligo	% inhibition of translation at conc of oligonucleotide	
name	10 μm	50 μm
Normal Phosphodiester Oligonucleotides		
LE 1.	47	54
LE 2.	43	33
LE 3.	33	40
LE 4.	30	24
LE 5.	35	52
LE 6.	22	63
LE 7.	43	58
LE 8.	75	21
LE 9.	74	92
LE 10.	32	27
Methyl phosphonate oligonucleotides		
LE 11.	< 5	85
LE 13.	> 95	67
Acridine tailed oligonucleotides		
LE 15.	27	68, 30, 87
LE 17.	< 5	< 5
LE 24.	35	81, 95
LE 28.	< 5	46
LE 30.	> 95	> 95
LE 34.	19	61, 60, 67
Cholesterol tailed oligonucleotide		
LE 32.	< 5	> 95
Controls for inhibition of Brome Mosaic Virus mRNA translation		
LE15		14
LE34		27

inhibitory or enhancing.

Inhibition of translation of RNA from the Leishmania species pathogenic to humans is shown in Table 3. We feel the data in these two tables show that the translation of targeted sequences in Leishmanial RNA from several species can be successfully inhibited in a sequence-specific manner.

The limit of confidence on the values in Tables 2 and 3 is currently no less than 30%. Note that LE 9 (35 nt oligonucleotide antisense to *L. enriettii* published splice leader sequence) is effective in other species of Leishmania (Table 3). The actual sequences

of the spliced leader in these species are being determined by us.

C. Inhibition of Growth of Cultured Leishmania

Table 3

Comparative effect of the normal phosphodiester 35 nucleotide antisense oligonucleotide (LE 9) on cell-free translation of RNA from three different species of Leishmania.

Species	% inhibition
L. Enriettii	92
L. Mexicana amazonensis	68
L. Braziliensis guyanensis	82

No activity was detected in any of the shorter oligonucleotides, regardless of the 5'-modification that they bore. The derivative bearing a cholesterol on the 5'-end (LE 32) gave similar results with or without pretreatment of cells with neuraminidase. Neuraminidase treatment was used to investigate whether or not sialic acid residues inhibit the uptake of oligonucleotides. Oligonucleotides LE 11, 24, 28, 29, and 30 were also assayed with and without pretreatment. As shown in Table 4, all oligonucleotides, except LE 24, gave no effect under both conditions. LE 24 at 100 μM decreased the population density by 80% after 48 hrs over placebo (H_20) controls. No difference with neuraminidase pretreatment was seen.

We have been interested in the possibility of delivering polyanionic oligonucleotides across cell membranes using liposomes, and have now done some preliminary experiments to explore feasibility of this approach. To test liposome uptake, liposomes with rhodamine incorporated were assayed on leishmanial cells for their ability to bind. Assays consisted of incubating three types of liposomes (neutral, positively and negatively charged) separately for 1-2 hours in the presence of leishmanial cells suspended in PBS. Cells were spun down and resuspended in normal growth media with 10% FCS. After resuspension and overnight incubation in media, fluorescent microscopy was used to visualize binding of the liposomes by appearance of a halo around the cells. In more than 99% of the cases, no halo was detected. Halos were not seen in free swimmers but were found in a few individuals in a cluster, but not in all clusters. A halo was also detected in an individual who was dividing. This may indicate that mature parasites may have a fully formed protective coat while the actively dividing or unmatured cells may have exposed binding sites for the liposomes.

We have assayed the ability of Lipofection[2] (an anionic lipid mix found to be effective in transforming certain cell lines) to enhance the cytotoxic effect of LE 24. The presence of the Lipofection reagent alone with *Leishmania* is not toxic. However, lipofection with LE 24 diminished the cytotoxicity of LE 24. Thus, Lipofection appears not to be useful for *Leishmania*. We are waiting for some liposomes of different composition from our collaborators to test for their fusion ability with *Leishmania*l cells.

[2]Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., and Danielsen, M. 1987. Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA* **84**:7413-7417.

Table 4

In vitro Cytotoxicity of Oligonucleotides to L. enriettii

			% Inhibition of Growth Compared to control (no oligo added)	
OLIGO	Length	[uM]	24 hr	48 hr
LE11	12	10	-5	10
		10	-38	-68
LE24	20	100	38	76
		100	45	78
LE28	14	100	-26	-12
		100	-10	-43
LE29	13	100	-12	-45
		100	8	-70
LE30	12	100	-	-34
		100	7	-62
LE32	12	10	23	19
		10	19	-16
		32	-4	-20
		32	2	-60
		100	36	-45
		100	16	-53
Sense Control with Acridine:				
LE16	12	100		0
		100		-20

The negative numbers indicate a larger population than the controls, thus non-effective targeting nucleotides may have some nutritional benefit.

Clearly, the longer oligonucleotide (LE24) is exerting a cytostatic or cytotoxic effect in cell culture, and demonstrates that antisense oligonucleotides of sufficient hybrid strength can, in fact, enter the cells and act. It must be noted, however, that the cell culture results are based on a limited number of experiments and should be repeated several times to quantitate the effect.

D. Sequencing the Spliced Leader

The dideoxy RNA sequencing of the New World strains of *L. mexicana amazonensis*, *L. braziliensis guyanensis*, and *L. donovani chagasi*, show that the spliced leader of our *L. enriettii* and the three other strains, using LE 33 as a primer, are identical in the first 15 nucleotides from the 5'-end, and that they are exactly those of *L. enriettii* as published. The hybridization of the 12 mer primer suggests that the targeted 12 nucleotides are also identical. The 5'-most nucleotide in all these sequences was not decipherable, in agreement with other published results. We are now confirming the sequence of the same area in

L. donovani. Based on our results it would appear that a single oligonucleotide sequence can be applied to all species of *Leishmania*. The conservation of the sequence gives a clear scientific rationale for investigation of the effects of antisense oligonucleotides on

Table 5

Sequencing Data for Leishmania sp. RNA

Published spliced leader sequence of L. enriettii:

3' GUU AUU UCA UGU CUU UGA CUA UGA AUA UAU CGC AA

Sequencing primer:

5' GT ACA GAA ACT G 3'

Nucleotides sequenced:

L. enriettii	AT ACT TAT ATA GCG T
L. braziliensis guyanensis	AT ACT TAT ATA GCG T
L. mexicana amazonensis	AT ACT TAT ATA GCG T
L. donovani chagasi	AT ACT TAT ATA GCG T

these human pathogen parasites.

IV. Summary

We have shown the following:

○ That oligodeoxynucleotides with acridines tethered to the 5'-position bind more tightly to target sequences than un substituted oligonucleotide.

○ That oligonucleotides complimentary to the spliced leader sequence of the mRNA of *Leishmania enriettii* inhibits translation of that mRNA, and that this inhibition is sequence-specific.

○ That a 20-nucleotide oligonucleotide complementary to this region inhibits the growth of *Leishmania enriettii* in cell culture.

○ That the spliced leader sequence of all *Leishmania enriettii* mRNAs is the same.

○ That the spliced leader of three L. species pathogenic to humans have the same sequence as *Leishmania enriettii*, and that their translation can be inhibited by our oligonucleotides.

We feel this demonstrates the feasibility of the ability of antisense oligonucleotides, complemenatry to the universal spliced leader of *Leishmania* mRNA, to arrest the growth of *Leishmania* in a sequence specific manner, and, in the Phase II of the project will develop the enhancement of potency necessary to produce a clinically useful drug.

APPENDIX 1

Leishmanial Oligonucleotides From *L.enriettii* Sequences:

Oligo name Generic	Descriptive	Sequence (5' TO 3')	Weight
Antisense Oligonucleotides			
Reverse Sequence of Spliced Leader:		3' GUU AUU UCA UGU CUU UGA CUA UGA AUA UAU CGC AA 5'	
LE1.	LET12[5',3']	T TAT ATA GCG TT	1.40
LE3.	LET12'[5',3']	CAA TAA AGT ACA	1.08
LE5.	LET16[5',3']	AT ACT TAT ATA GCG TT	2.54
LE7.	LET20[5',3']	ACT GAT ACT TAT ATA GCG TT	2.55
LE9.	LET35[5',3']	CAA TAA AGT ACA GAA ACT GAT ACT TAT ATA GCG TT	1.25
LE11.	LET12[5',MP,3']	T TAT ATA GCG TT	0.15
LE13.	LET12'[5',MP,3']	CAA TAA AGT ACA	0.33
LE15.	LET12[5',Ac,3']	Ac T TAT ATA GCG TT	1.04
LE17.	LET12'[5',Ac,3']	Ac CAA TAA AGT ACA	0.81
LE22.	LET35[5',6N,3']	6N CAA TAA AGT ACA GAA ACT GAT ACT TAT ATA GCG TT	1.17
LE24.	LET20[5,'Ac,3']	Ac ACT GAT ACT TAT ATA GCG TT	3.8
LE28.	LEHS14[5'Ac,3']	Ac AA ACT GAT ACT TAT	1.05
LE30.	LEC12[5'Ac,3']	Ac GT ACA GAA ACT G	1.06
LE32.	LECHT12[5',CH,3']	Ch T TAT ATA GCG TT	0.66
LE33.	LEC12[5',3']	GT ACA GAA ACT G	0.98
LE34.	LET16[5,Ac,3']	Ac AT ACT TAT ATA GCG TT	1.52
Sense Oligonucleotides:			
Spliced Leader:		5' AAC GCU AUA UAA GUA UCA GUU UCU GUA CUU UAU UG 3'	
LE2.	LEAT12[5',3']	AAC GCT ATA TAA	1.40
LE4.	LEAT12'[5',3']	T GTA CTT TAT TG	0.97
LE6.	LEAT16[5',3']	AAC GCT ATA TAA GTA T	1.12
LE8.	LEAT20[5',3']	AAC GCT ATA TAA GTA TCA GT	2.67
LE10.	LEAT35[5',3']	AAC GCT ATA TAA GTA TCA GTT TCT GTA CTT TAT TG	2.89
LE12.	LEAT12[5',MP,3']	AAC GCT ATA TAA	
LE14.	LEAT12'[5',MP,3']	T GTA CTT TAT TG	
LE16.	LEAT12[5'Ac,3']	Ac AAC GCT ATA TAA	2.23
LE18.	LEAT12'[5'Ac,3']	Ac T GTA CTT TAT TG	2.01
LE23.	LEAT35[5',6N,3']	6N AAC GCT ATA TAA GTA TCA GTT TCT GTA CTT TAT TG	1.23
LE25.	LEAT20[5'Ac,3']	Ac AAC GCT ATA TAA GTA TCA GT	3.3
Other Oligonucleotides:			
LE26.	LESP12[5'Ac,3']	Ac GCA TAC CAA TAA	1.41
LE27.	LEASP12[5'Ac,3']	Ac TTA TTG GTA TGC	1.08
LE29.	LEU213[5'Ac,3']	Ac ATA AGT ACA GTT T	1.05
LE31.	LESQ18[5',3']	GAA GAC AGG ITC CGG AAG	1.87
LE35.	LEP4[5',3']		

Key

LE indicates oligonucleotide for *L. enriettii*,
T indicates oligonucleotide with antisense sequences.
AT indicates oligonucleotide with sense strand sequences.
# indicates length of oligonucleotide.
having #' indicates oligonucleotide to 3' portion of spliced leader.
without ' indicates oligonucleotide to 5' portion of spliced leader.
[5',3'] indicates the direction of reading sequence.

MP indicates methylphosphonate linkages within oligonucleotide except at 5' most linkage which is a normal phosphodiesterase bond.
Ac indicates acridine intercalater at 5' end.
6N indicates hexylamine tail at 5' hydroxyl.
SP indicates antisense sequences spanning medRNA splice site.
ASP indicates sense sequences spanning medRNA splice site.
U2 indicates antisense sequences to LE's nsRNA U2 in region of potential binding region with SL.
C indicates antisense sequence to a conserved sequence in all confirmed Kinetoplastidae SL.
HS indicates antisense sequence to the hypothetical U2 binding region in SL.
SQ indicates oligonucleotide to the intron portion of the medRNA.
Ch indicates a 5' cholesterol tail.
[SL = Spliced Leader sequence of mRNA]